Studies on the In Vivo Formation of Acrolein: 3-Hydroxypropylmercapturic Acid as an Index of Cyclophosphamide (NSC-26271) Activation[1,2]

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SUMMARY

3-Hydroxypropylmercapturic acid (MCA) has been quantitatively determined in the urine of rats given cyclophosphamide (CP), related antineoplastic agents, allyl alcohol, or acrolein, with a simple procedure involving the use of an amino-acid analyzer. Male rats (300-400 g) injected with CP (50 mg [179.1 μmols]/kg of body weight) excreted 16.7 μmols of MCA/kg in their 24-hour urine. Equivalent amounts of isophosphamide produced 9.0; triphosphamide, 16.1; ASTA-5607, 7.2; ASTA-5122, 4.1; and cytoxyl alcohol, 0.4 μmols of MCA/kg. From allyl alcohol and acrolein, 26.3 and 19.7 μmols of MCA/kg were obtained respectively. MCA values were directly proportional to drug dose levels. Since acrolein and phosphorodiamidic acid mustard are the toxic decomposition products of aldophosphamide, and acrolein conjugation with glutathione appears to be the first step for MCA formation, values for MCA would reflect active CP levels. The in vitro interaction of acrolein with glutathione, other sulfhydryl compounds, and a few amino acids at concentrations of 0.15 μmols/ml was also studied. The decrease of acrolein's main absorption peak at 209 nm was used to follow its reaction rate. The faster interactions observed were with the sulfhydryl compounds, where a 50% decrease of absorption in interactions with glutathione and cysteine (at pH 7.4 and 23°C) took place in 111 and 30 seconds respectively. Incubation of these adducts at 37°C and 100°C generated acrolein with a maximum recovery yield of 83% at 100°C. Five patients given 1 g of CP iv excreted 6.4-50 μmols of MCA in their urine in 6 hours.

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The formation of acrolein from cyclophosphamide (CP) and related antineoplastic agents during their in vitro oxidation, whether by liver microsomes or by simple chemicals, was shown in our laboratories in 1971 (1) and confirmed in several others (2-5). The search for acrolein as a possible product of CP was based on the similarities existing between the structures of oxidized polyamines (unstable acrolein-generating compounds [6-8]) with the hypothetical primary metabolites advanced by Brock and Hohorst (9) and Norpoth et al (10). The finding of acrolein brought with it implications that played an important role in the elucidation of the metabolic pathway of CP. It strongly supported the existence of the hypothetical primary metabolites. It made apparent that elimination of acrolein from either of these structures, whether formed by *O*-dealkylation or *N*-dealkylation, left phosphorodiamidic acid mustard as the residual part of the molecule. This latter compound, a nitrogen mustard synthesized with several others by Friedman et al (11), was already known to be an effective antitumor agent in experimental animals (12) but rather ineffective in clinical trials (13).

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[2]3-Hydroxypropylmercapturic acid: CAS reg. No. 23127-40-4.

Cyclophosphamide: CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate.

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An indirect identification of aldophosphamide was advanced by Hill et al in 1972 (14), and the conclusive identification of its semicarbazone derivative was achieved recently (15). In 1973 Colvin et al (2) and Connors et al (4) independently identified the phosphorodiamidic acid mustard as a CP degradation product in vitro, and more recently its in vivo detection was successfully accomplished (16).

In the meantime, in a series of abstracts and in a detailed paper, Kaye's group (17-20) reported the presence of 3-hydroxypropylmercapturic acid (MCA) in the urine of rats given allyl alcohol, several allyl compounds, or acrolein. This compound was isolated as a dicyclohexylammonium salt from the combined urine of a large number of rats (144 received acrolein). Acrolein appeared to be the required intermediate, formed in vivo from the allyl compounds, that conjugates with glutathione to initiate the metabolic chain of reactions leading to the MCA formation. Gray and Barnsley (21) proposed a similar suggestion to explain the resulting MCA after administration to experimental animals of crotyl compounds or crotonaldehyde. More recently, Kaye and Young (22) reported in a brief paper the qualitative detection of MCA in the urine of rats and patients given CP.

Work in our laboratories confirmed the initial finding by Kaye (20) on the presence of MCA in the urine of rats given acrolein, and we independently detected this MCA in the urine of rats given CP. The development of a procedure that could be used to quantitate this MCA in the urine of individual experimental animals or man took first priority. This was considered important and potentially valuable to pharmacologic studies on CP since MCA values were expected to be proportional to the fraction of CP metabolized through its active pathway. Neither acrolein nor MCA would be formed from the inactive metabolites of CP.

The purpose of this paper is to report this procedure and the MCA amounts excreted by individual experimental animals given CP, a series of related antineoplastic agents, allyl alcohol, and acrolein. Also, a preliminary report on the amounts of MCA excreted by a small group of patients receiving CP is included, as well as in vitro studies related to the first accepted step in MCA formation, the conjugation of acrolein with glutathione.

MATERIALS AND METHODS

Drugs.—CP was purchased from Mead and Johnson, Evansville, Ind. Isophosphamide (IP), triphosphamide, and cytoxyl alcohol were obtained from Dr. A. R. Stanley, Drug Development Branch, Drug Research and Development Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md. ASTA-5607 and ASTA-5122 were made available by the courtesy of Dr. N. Brock, Pharmacology Laboratories, Asta-Werke AG, Brackwede, Germany.

Chemicals.—Acrolein, reagent grade, was purchased from Eastman Kodak Co, Rochester, NY. *S*-(3-hydroxypropyl)-L-cysteine (3-H Pr Cyst) and *S*-(2-hydroxypropyl)-L-cysteine were synthesized according to Barnsley (23). MCA, as its dicyclohexylammonium salt, was synthesized according to the procedure reported by Kaye et al (24). All other chemicals were obtained from commercial sources in the highest degree of purity available.

Acrolein-sulfhydryl compound adducts.—These adducts were prepared by mixing 15 μmols of acrolein with equivalent amounts of the sulfhydryl compound in 100 ml of 0.02 M phosphate buffer (Na_2HPO_4-KH_2PO_4) at pH values of 7, 7.4, and 8.5. The mixtures were left at room temperature (23°C-25°C) for various lengths of time (see Results).

Adduct reduction.—Two mg of sodium borohydride in 0.2 N NaOH (0.2 ml) was added to 8-ml solutions (pH 7.4) of acrolein with the sulfhydryl compound (2.5 μmols/ml) after incubation at room temperature for 25 minutes, and left for 1 hour undisturbed before starting identification procedures.

Iodometric titrations.—Glutathione and acrolein-sulfhydryl compound adducts were titrated according to Woodward and Fry (25).

Procedures for acrolein regeneration.—Two sets of experiments, at 37°C and 100°C, were carried out to study the regeneration of acrolein from the above adducts. The procedures used were essentially similar to those formerly used for oxidized CP (1). The above adducts were contained in 250-ml two-necked round-bottomed flasks either in a 37°C waterbath or on heating mantles. One of the openings was the inlet for the carrier gas (air, 130-140 ml/min) and the other (outlet) was connected to a cooled 400-mm West condenser whose upper end was closed by an adaptor connected by rubber tubing to a gas absorption tube (the cold trap). This trap, containing 150 ml of water, was surrounded by crushed ice and served to trap and accumulate any released acrolein carried by the air bubbling through it. The trapping efficiency of these cold traps for 15 μmols of acrolein in 100-ml 0.02 M phosphate buffer solutions (pH 7.4) at 100°C was 80%-90%.

Determination of acrolein.—Acrolein was determined in the cold trap solutions by fluorometry as well as by ultraviolet spectrometry as described in detail in our previous studies (6,26,27). Ultraviolet spectra were recorded in the 200-250-nm region where the main absorption peak of acrolein occurs

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(209 nm). The decrease of this peak was followed as an index of the reaction rate of acrolein with the diverse adduct components studied. A Unicam SP-800 (Phillips Electronics Instruments, Mt. Vernon, NY) was used.

Experiments with animals.—Male rats (300-400 g, Kx strain, obtained from the New England Deaconess Hospital stock) were used in these studies. CP and related drugs were administered ip. Acrolein and allyl alcohol in peanut oil were given sc after light ether anesthesia. After the doses were given the rats were placed in metabolic cages and urine was collected for 24 hours in bottles surrounded by crushed ice.

Paper chromatography studies.—Ascending chromatograms using Whatmann 3 MM paper in an acidic solvent mixture (*n*-butanol:water:acetic acid, 12:5:3 by vol) and in a basic one (*n*-butanol:pyridine:3 M NH_3, 80:20:1 by vol) were developed for 8-9 hours. Concentration of the urine by methyl ethyl ketone extraction and the rest of the procedures to detect sulfur-containing compounds with chloroplatinic acid reagent (28) and amino acids with ninhydrin, followed the guidelines suggested by Kaye (20).

MCA quantitation method.—(*a*) Hydrolysis procedure: The urine from treated or control rats was filtered on paper, and the aliquots were subjected to hydrolysis in sealed ampules at a 2 N HCl molarity at 100°C for 3 hours. Authentic MCA in aqueous solutions or in normal urine was similarly processed; 0.2 or 0.4 ml of the hydrolysate added to 1.8 or 1.6 ml of 0.2 M sodium citrate buffer (pH 2.2) and the mixture injected into the amino-acid analyzer. (*b*) Amino-acid determinations: Amino-acid analyses were performed in a Beckman Amino-Acid Analyzer Model 120 C. Most of the hydrolyzed urine analyses were carried out with the acid-neutral column, using 0.2 M sodium citrate buffer, pH 3.18, following routine procedures described for physiologic fluids (29). Authentic 3-H Pr Cyst was used to prepare the standard solutions.

Patient studies.—The small number of patients receiving CP and studied thus far for the presence of MCA in their urine, were unselected with regard to concurrent treatments or type of disease.

RESULTS

Qualitative Determination of MCA

The presence of MCA in the urine of rats given acrolein was confirmed in a series of preliminary experiments following the procedures reported by Kaye (20). The 24-hour urine collected from four rats (330-350 g in weight, Kx strain) injected sc with 150 μmols of acrolein in peanut oil, was extracted with methyl ethyl ketone, and the extract was evaporated to dryness, dissolved in 2 ml of methanol, and paper chromatographed in acidic and basic solvent systems (see Methods). The chromatograms were stained with the chloroplatinic acid reagent, used to detect sulfur-containing compounds (28), and with ninhydrin. As expected, one white spot appeared with the chloroplatinic acid, but none with ninhydrin. The R_f values on ascending chromatograms were 0.72 in the acidic solvent and 0.40 in the basic one. Acid hydrolysis of this compound resulted in a secondary product that gave a white spot with the chloroplatinic reagent and a blue one with ninhydrin and had an R_f value of 0.23 in the first solvent and a value of 0.28 in the latter one. Subsequently, two rats (360 g) were injected with 30 mg of CP ip, and the urine extracted as above. This new extract and its acid hydrolysis product were chromatographed alongside the former compounds obtained from the acrolein-treated rats, and with authentic MCA and 3-H Pr Cyst. Identical staining characteristics and R_f values were obtained for the three pairs of compounds. Furthermore, as expected, the acid hydrolysis of the synthetic MCA resulted in the formation of 3-H Pr Cyst.

Having established qualitatively that MCA was present in the urine of rats given acrolein or CP, efforts were directed to develop a procedure to quantitate MCA in individual experimental animals. Use of an amino-acid analyzer to measure the hydrolytic product of MCA (3-H Pr Cyst) appeared feasible and the following experimental basis was established.

a. Synthetic 3-H Pr Cyst gave only one peak in the amino-acid analyzer chromatogram in the region corresponding approximately to 130 minutes of elution time using the acid-neutral column. Continuing the chromatogram with the basic column, no additional peaks were detected.

b. Synthetic MCA hydrolyzed for 3 hours in 2 N HCl in sealed ampules at 100°C resulted in the formation of equimolar amounts of 3-H Pr Cyst.

c. Synthetic MCA added to normal rat urine and similarly hydrolyzed behaved as above, and most important, no constituent from the hydrolyzed urine interfered with the 3-H Pr Cyst peak (fig 1). This peak appeared after serine (approximately 30 mins) and before glutamic acid (approximately 15 mins). Proline, a small peak 5 minutes after 3-H Pr Cyst, disappeared when higher concentrations of this latter compound were recorded.

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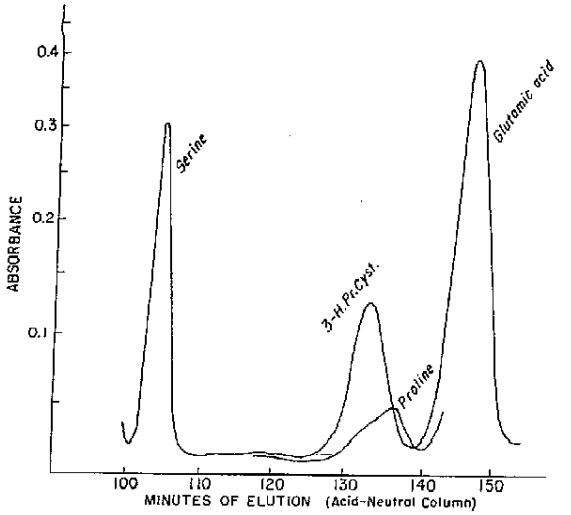

FIGURE 1.—Amino-acid analyzer chromatogram of hydrolyzed urine from a rat (355 g) given 93 μmols of CP (75 mg/kg rat body weight). MCA measured in 24-hr urine, 9.65 μmols (10.3% yield).

d. Urine from untreated rats before or after hydrolysis did not show any inflection in the chromatograms corresponding to 3-H Pr Cyst.

e. *S*-(2-hydroxypropyl)-L-cysteine appeared in the chromatogram approximately 40 minutes before its isomer.

Quantitation of MCA in Urine of Experimental Animals

The procedure finally devised involved acid hydrolysis and the use of the amino-acid analyzer (*see* Methods). Following this method, the amounts of MCA represented in figure 2 were obtained. MCA was measured in rat urine collected during the first 24 hours after administration of CP, isophosphamide (IP), or acrolein, at diverse dose levels. Three male rats (300-400 g) were used at each level. The dose range of acrolein was from 50 to 300 μmols/kg rat body weight. For the two drugs, a rather similar dose range was investigated: between 12.5 and 75 mg/kg body weight for CP and equivalent amounts (except for the lowest dose) for IP. The amounts of MCA excreted were directly proportional to the amounts of drug given. The average percentage yields of MCA from CP (9.7%) and IP (4.7%) remained practically constant at all dose levels. For acrolein, however, the yields varied from 17.6% for the lowest doses to 10% for the higher ones, where wider individual variation appeared.

Several related antineoplastic agents were compared for their ability to produce MCA at a dose

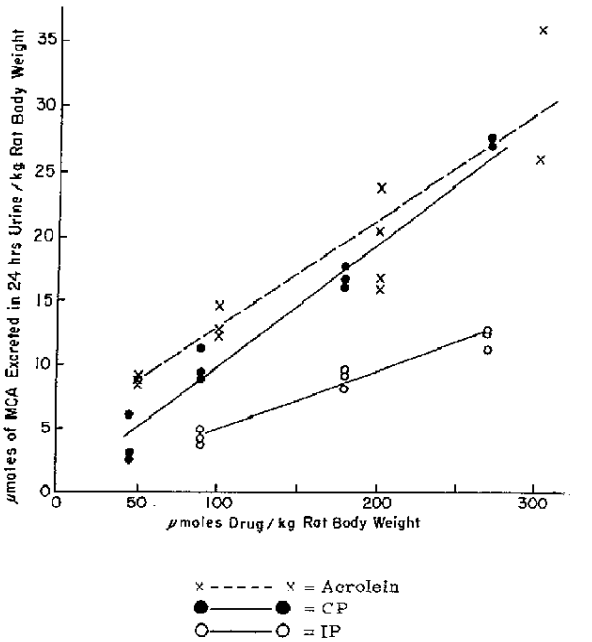

FIGURE 2.—Comparison of the amounts of MCA excreted by rats given acrolein, CP, and IP.

level of 179.1 μmols/kg rat body weight, a dose which is equivalent to 50 mg/kg of CP. Among the drugs listed in table 1, the highest yields of MCA were given by CP followed by triphosphamide; cytoxyl alcohol gave the lowest amounts of MCA. The most effective compounds in the formation of MCA studied to date, however, are allyl alcohol followed by acrolein. Also forming part of table 1 are the in vitro amounts of acrolein generated from 20 μmols of these drugs during the first 2 hours of oxidation with modified Fenton's reagent at 37°C. MCA and acrolein values (for this short period of oxidation studied) appear to correlate well only for CP and IP and to a lesser degree for cytoxyl alcohol. For the rest of the compounds, no correlations are apparent. Two rats injected with 5 and 10 μmols of MCA/rat excreted 60% and 65% respectively of this presumed end product in their 24-hour urine.

Quantitation of MCA in Human Urine

Using identical methods as described for the experimental animals, aliquots of urine were hydrolyzed and subjected to amino-acid analysis. The five patients studied were unselected and were receiving treatment with multiple drugs. The main

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TABLE 1.—*Comparison on the in vivo formation of MCA and in vitro formation of acrolein, from CP and related compounds*

Compound injected (179.1 μmols/kg*)	MCA (μmols/kg) in first 24-hr urine	Yield (%)	Acrolein collected (μmols) from 20 μmols of compound after 2-hr oxidation†	Yield (%)
CP	16.7	9.3	2.72	13.6
IP	9.1	5.1	1.57	7.8
Triphosphamide	16.1	8.9	1.35	6.7
ASTA-5607	7.3	4.0	2.06	10.3
ASTA-5122	4.1	2.3	1.95	9.7
Cytoxyl alcohol	0.4	0.2	0.58	2.9
Acrolein	19.7	11.0		
Allyl alcohol	26.3	14.7		

*Equivalent to 50 mg of CP/kg rat body wt. Three rats were used in each experiment.

†Modified Fenton's reagent (hydrogen peroxide, 266 μmols; ferrous sulfate, 30 μmols; edetic acid, 53 μmols; 0.2 M phosphate buffer, pH 7; final vol, 10 ml), 37°C.

objective was to test the feasibility of this method to measure MCA in man. All patients received 1 g of CP iv, and their urine was collected for 6 hours.

The amounts of MCA varied widely. The lowest levels found were 6.4 and 9.9 μmols, one intermediate value was 17.5 μmols, and the highest values found were 40 and 50 μmols. If the dose of CP given was expressed in mg/kg, the dosage fluctuated between 10 and 18 mg/kg. No correlation between dose values and amounts of MCA was apparent.

In Vitro Studies on Acrolein-Sulfhydryl Compound Adducts

According to the extensive literature on MCA formation (30), the first step in the series of reactions leading to MCA would be the conjugation of acrolein with glutathione. Taking advantage of the high molecular extinction coefficient of acrolein at 209 nm (11,540), it was thought that its conjugation with glutathione, postulated to occur at the acrolein double-bond reactive site, could be followed by recording the decrease of absorbance at this wavelength as the reaction proceeded. The concentration of acrolein chosen was 0.15 μmols/ml 0.02 M phosphate buffer, whose spectrum in the Unicam SP-800 with an absorbance scale up to 2.0, gave an absorbance maximum around 1.72. A similar concentration of glutathione gave at this wavelength an absorbance value of only 0.6. Mixing equimolar amounts of both compounds and incubating the mixture at room temperature (23°C), the expected decrease took place in a short period of time (fig 3). In table 2 the time periods are recorded in which a 50% decrease occurred when acrolein interacted with diverse sulfhydryl compounds at room temperature and at three different

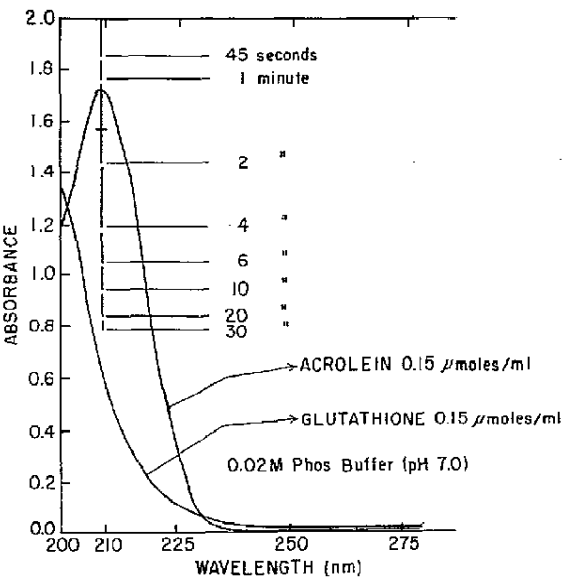

FIGURE 3.—Decrease of acrolein absorption at 209 nm following interaction with equimolar amounts of glutathione.

TABLE 2.—Decrease of acrolein's absorbance by sulfhydryl compounds*

	Time period (secs) in which 50% decrease of the 209-nm absorption peak occurred		
	pH 7	pH 7.4	pH 8.5
Glutathione	111	58	17
Acetylcysteine	345	165	57
Cysteine	74	30	4-5

*Reactions carried out at room temperature (23°C-25°C). Concentration of reactants, 0.15 μmol/ml 0.02 M phosphate buffer (Na_2HPO_4-KH_2PO_4).

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TABLE 3.—Decrease of acrolein's absorbance by some amino acids*

	Decrease of 209 nm peak (%)	Time incubated at room temperature (mins)
Glycine	10	40
Arginine	8	45
Serine	2	45
Methionine	0	15

*Concentration of acrolein and amino acids, 0.15 μmol/ml 0.02 M phosphate buffer, pH 7.4.

pH values. The fastest reaction observed was the interaction of acrolein with cysteine at pH 8.5. In interactions of acrolein with amino acids with other functional groups, the decrease in absorbance was very slow (table 3).

Iodometric titration of the sulfhydryl compounds (25) before and after interaction with acrolein further confirmed that the reaction took place at the sulfhydryl group site in these compounds.

Adduct Decomposition With Regeneration of Acrolein

It is assumed that conjugation of acrolein with glutathione takes place by the addition of the nucleophilic glutathione anion to the β carbon of the allylic double bond of acrolein (20,31). Reactions where glutathione is substituted for acetylcysteine or cysteine would be expected to take place by a similar mechanism. The products of these reactions would be 3-substituted propanals, a well-known group of acrolein-generating compounds (7,27).

Following procedures formerly used to collect acrolein during in vitro oxidation of spermine or CP (1,6), the above adducts, incubated at 37°C and 100°C, similarly generated this aldehyde. Air was used as the carrier gas to remove the regenerated acrolein into cold-water traps where it was collected by absorption. In table 4 are listed the amounts of acrolein collected from these incubations during two time periods at each temperature. In the experiments at 100°C all the acrolein that could be recovered (83% yield) was collected during the first 40 minutes. Reference solutions of acrolein produced 88% yield under similar experimental conditions. The 2-hour periods in the incubations at 37°C were arbitrarily chosen and the amounts of acrolein collected do not represent the maximal amounts that can be obtained at these temperatures.

3-Substituted propanals can be stabilized by their reduction to the corresponding alcohols. This procedure was employed for the identification of the aminoaldehydes of spermine, using sodium borohydride as the reducing agent (32). A similar method was applied to the acrolein-acetylcysteine and acrolein-cysteine adducts. The expected alcohols, if 3-substituted propanals had been formed, were MCA and 3-H Pr Cyst respectively, ie, the same compounds already obtained from the urine of rats given acrolein. This did occur, and both compounds were detected by paper chromatographic techniques and quantitated with the amino-acid analyzer. Preliminary measurements indicate that reduction of the acrolein-acetylcysteine adduct resulted in the formation of MCA in a 49.6% yield, and reduction of the acrolein-cysteine adduct re-

TABLE 4.—Acrolein generation from acrolein-sulfhydryl compound adducts*

	Temperature				
	37°C		100°C		
Adduct	Incubation period (hrs)	Acrolein collected† (μmols)	Incubation period (mins)	Acrolein collected† (μmols)	Maximal yields (%)
Glutathione-acrolein	1	0.55	10	7.50	
	2	1.20	40	12.30	82
Acetylcysteine-acrolein	1	0.13	10	5.65	
	2	0.44	40	12.40	83
Cysteine-acrolein	1	3.20	10	7.40	
	2	5.50	40	12.50	83
Acrolein (buffered reference solution)	1	7.10	10	11.50	
	2	9.80	40	13.20	88

*Adduct formation = 15 μmols of each compound in 100 ml of 0.02 M phosphate buffer (pH 7.4), incubated at room temperature (23°C-25°C) for 1-2 hrs.

†Carrier gas = air 130-140 ml/min. Acrolein collected in 150-ml cold-water traps.

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sulted in formation of 3-H Pr Cyst in a 21.6% yield. If further confirmed, these results would indicate that the addition reaction to acrolein's double bond took place, but that at the same time another form of reversible reaction, judging from the 83% recovery of acrolein from the original adducts, most likely occurred. The glutathione acrolein adduct has not yet been similarly studied.

DISCUSSION

This paper on the identification and quantitation of MCA in the urine of rats given CP, related drugs, allyl alcohol, or acrolein, added to the basic work by Kaye's group (17-20) and Gray and Barnsley (21), and to the physiopathologic studies on the hepatotoxicity of allyl alcohol (33-35), led, with a high degree of certainty, to the conclusion that acrolein is generated in vivo from all these compounds as it is in vitro. Acrolein, as already considered by others (20,21), appears to be the required intermediate for the above MCA formation.

Consequently, MCA presence in the urine of rats or man given CP indicates that the active metabolite aldophosphamide decomposes in vivo with the generation of acrolein, as it does in vitro (3-5,27). Thus, the levels of this MCA will be directly proportional to the fraction of aldophosphamide that escaped inactivation to carboxyphosphamide, as well as to the amount of phosphorodiamidic acid mustard formed. Structurally, this latter active metabolite can be considered as the residual part of aldophosphamide once acrolein is eliminated. It follows that the MCA quantitation indirectly measures "active CP" (fig 4). This is an important consideration since methods most commonly used to follow CP activation in vivo and attempts to assess potential pharmacologic modifications of this activation have relied heavily on the nonspecific alkylation of 4-(*p*-nitrobenzyl)pyridine (36), with alkylation values usually expressed in relation to a standard, such as *nor*-nitrogen mustard (37).

There is an awareness among investigators that the diverse alkylating metabolites derived from CP may possess different molecular extinction coefficients, that the alkylation values obtained are the sum of the individual ones, and more important, that alkylating activity does not necessarily indicate antitumor or cell growth inhibitory activities. For example, although carboxyphosphamide, in itself, does not possess significant antitumor activities and apparently is nontoxic in vivo, nevertheless, it is as good an alkylating agent as aldophosphamide (37). This latter property makes it difficult to estimate the aldophosphamide to carboxyphosphamide ratio in any given alkylation measurements in blood samples or other organic fluids. This has been a serious limitation to many studies attempting to induce or inhibit CP activation. For example, it is known that the total concentration of alkylating activity in the blood (C) multiplied by time (T) remained relatively constant for a given CP dosage. In one clinical study where a deliberate attempt was made to potentiate CP activity in one patient by concurrent administration of an aldehyde oxidase inhibitor, the C × T product obtained was not higher than that of other patients receiving the same doses of CP (38). This observation, unfortunately, appears to have discouraged further clinical studies along this line of research. The above results were to be expected since C includes the alkylating activity of aldophosphamide, carboxyphosphamide, and others. Consequently, even if the drug combination succeeded in increasing the aldophosphamide to carboxyphosphamide ratio, the alkylation measurements alone could not have detected it. MCA quantitation in this patient's urine, on the other hand, might have detected this change as suggested by the experimental data presented in this paper, a possibility which,

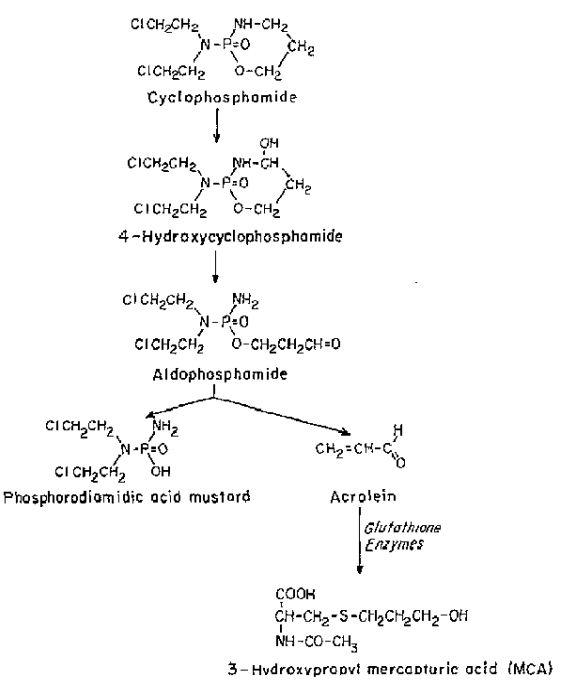

FIGURE 4.—Schematic representation of the main active metabolic pathway of CP with one of its end products, acrolein, as the MCA precursor.

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together with that of the presence of MCA precursors in the blood, will be subsequently investigated.

Speculations on the mechanism of action of the acrolein formed in vivo or administered suggest that compounds possessing accessible sulfhydryl groups might be acrolein's main target. The reaction rate of acrolein with these compounds, as observed in vitro, is far above that with compounds possessing amino or hydroxyl groups. Barring unexpected enzymatic catalyses, it appears that this modality of action might also take place in vivo. The reaction of acrolein with available sulfhydryl groups of enzymes has been described (39, 40), as well as the decrease of acrolein cytotoxicity in vitro by glutathione (41). It is most likely that glutathione in the cells is the main (although not necessarily the most significant) target of acrolein, judging from (*a*) the resulting MCA yields, (*b*) the glutathione ubiquity and concentration in diverse tissues (42,43), (*c*) the existence of glutathione alkene-transferase (31) which might catalyze in vivo this already fast interaction, and (*d*) the decrease in glutathione levels in the liver and kidney of experimental animals given acrolein as observed by us in preliminary experiments.

Considering the large variations of MCA excretion in the few patients studied so far, possible correlations that might be responsible for these variations will be searched for as soon as a significantly large number of patients receiving CP are studied. Since the patients reported here received multiple-drug therapy, it is conceivable that the highest levels measured might be the result of a non-planned stimulation of CP activation. Because of the small number of observations it is not possible at this moment to advance an explanation for the MCA values found.

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